Immunohistochemical Studies of Atypical Conjunctival Melanocytic Nevi

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Objective: To evaluate with immunohistochemical methods 5 atypical melanocytic conjunctival lesions.

Methods: This was a retrospective clinicopathologic study. Routine histochemical staining was performed with multiparametric immunohistochemical analysis with monoclonal antibodies immunoreacted on paraffin sections to identify the following cell antigens: S-100, MART-1, HMB-45, CD45, CD68, CD1a, lysozyme, and Ki-67 (nuclear proliferation protein).

Results: A unique granular cell nevus contained periodic acid–Schiff–positive, diastase-resistant granules and immunoreacted with monoclonal antibodies against S-100 protein and melanocytic-associated antigens MART-1 and HMB-45. Results for CD45, CD1a, CD68, and lysozyme immunostaining of the granular cells were negative. Two epithelioid cell (clonal or inverted) nevi exhibited an identical immunohistochemical profile. Only the balloon cell nevus was MART-1–positive and HMB-45–negative. The granular cell and blue nevi immunoreacted negligibly with Ki-67 (approximately 1% of cells).

Conclusions: S100 and MART-1 reliably immunostained all nevocytic morphologic variants. HMB-45 immunoreactivity of the granular, epithelioid/clonal, and blue nevi did not indicate a more active or proliferative lesion but instead suggested abnormal melanogenesis. Ki-67 was the most valuable immunohistochemical adjunct to morphology for the diagnosis of these benign variant conjunctival nevi, because melanomas display a much higher proliferation index (>10% nuclear positivity among all cells counted) than the current nevi (approximately 1%).

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METHODS

From the regular and consultation files of the David G. Cogan Laboratory of Ophthalmic Pathology at the Massachusetts Eye and Ear Infirmary, we retrieved conjunctival lesions that had been diagnosed as atypical nevus, nevus with unusual features, borderline nevus, balloon cell nevus, or blue nevus from 2005 to 2008. Hematoxylin-eosin–stained slides were critically reexamined. Five lesions were judged...
appropriate for inclusion in this study and further evaluation. While all were small specimens, only 1 case (blue nevus) had insufficient archival tissue embedded in paraffin blocks for additional sectioning and staining. Routine histochemical staining included periodic acid–Schiff with and without diastase, Masson trichrome, and reticulin. Immunohistochemical staining with the immunoperoxidase method was conducted using the monoclonal antibodies and their targeted antigens listed in Table 1.

The staining was done on BenchMark XT automated tissue staining systems (Ventana Medical Systems, Tucson, Arizona) using validated protocols. Endogenous peroxidase activity was blocked with H₂O₂ before antibody incubation. A combination of EDTA and boric acid in Tris buffer (CC1 reagent; Ventana Medical Systems) was applied to the tissue sections for antigen retrieval as needed, and the process was carried out prior to primary antibody incubations. The tissues were washed and incubated with the primary antibodies indicated above, followed by incubation with UltraView horseradish peroxidase–conjugated multimer antibody reagent. Antigen detection was performed using UltraView and diaminobenzidine as the chromogen, which yields a brown/black reaction product. In case 3, aminoethylcarbazole was used to produce a red staining result. Tissues were counterstained with hematoxylin. Bleaching of melanin followed by immunoperoxidase staining was not conducted because of the frequent loss or partial dissolution of paraffin sections in our experience.

None of the lesions in this series was heavily pigmented and all had a significant proportion of tumor cells that were either very lightly pigmented or wholly nonpigmented. Macrophages filled with phagocytosed melanin (melanophages) were present in all lesions in small to moderate numbers. They could easily be distinguished from the lightly pigmented predominant tumor cells by virtue of their coarsely clumped cytoplasmic melanin granules that totally obscured the nucleus. Consequently, it was easy to discern positive immunostaining, which was a deep brown/black with chromogen diaminobenzidine and evenly and diffusely distributed in the positive cells, compared with the fainter brown coloration conferred by the native melanization of the tumor cells. In case 3 of the new blue nevus, the red chromogen aminoethylcarbazole was used to highlight positivity, obviating any possible confusion with melanin. The immunolabeling was evaluated impressionistically for the selected melanocytic antigens and graded. Counting of positive nuclei labeled with Ki-67 was conducted in 2 high-power fields (×400) using a 10×10 reticle. This result was also stated as a proliferation index, that is, the percentage of positive cells in the 2 lesions in which any positivity was found (only the granular and blue nevi).

Clinical information, including detailed ocular histories and clinical photographs when available, were reviewed and analyzed in light of the histomorphologic immunohistochemical findings and the clinical follow-ups. The immunohistochemical data were compared with those previously published in the ophthalmic and dermatologic literatures.

Table 1. Summary of Immunologic Probes

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen for</th>
<th>Specificity</th>
<th>Source</th>
<th>Dilution</th>
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<tr>
<td>S100</td>
<td>Cytoplasm of melanocyte, Schwann cell, glial cell, among other cell types</td>
<td>Mouse monoclonal, IgG2a</td>
<td>Prediluted</td>
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<td>MART-1</td>
<td>Melanocytic cytoplasmic premelanosomes</td>
<td>Mouse monoclonal, IgG2b</td>
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<td>HMB-45</td>
<td>Melanocytic cytoplasmic premelanosomes</td>
<td>Mouse monoclonal, IgG1</td>
<td>1:100</td>
<td></td>
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<tr>
<td>PNL-2</td>
<td>Melanocytic and polymorphonuclear leukocyte cytoplasm</td>
<td>Mouse monoclonal, IgG1</td>
<td>Prediluted</td>
<td></td>
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<tr>
<td>CD45</td>
<td>Lymphocytic, histiocytic, hematopoietic cell membranes</td>
<td>Mouse monoclonal, IgG1</td>
<td>Prediluted</td>
<td></td>
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<tr>
<td>CD1a</td>
<td>Langerhans cell membrane</td>
<td>Mouse monoclonal, IgG1</td>
<td>Prediluted</td>
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<tr>
<td>CD68</td>
<td>Monocyte/histiocytic cytoplasm</td>
<td>Mouse monoclonal, IgG1</td>
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<tr>
<td>Lysozyme</td>
<td>Monocyte/histiocytic cytoplasm</td>
<td>Mouse monoclonal, IgG1</td>
<td>Prediluted</td>
<td></td>
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<tr>
<td>Ki-67</td>
<td>Nuclear proliferation protein expressed in S phase</td>
<td>Rabbit monoclonal, IgG</td>
<td>Prediluted</td>
<td></td>
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</table>

aVentana Medical Systems, Tucson, Arizona.
bSignet Laboratories, Dedham, Massachusetts.
cDako Corporation, Carpinteria, California.

RESULTS

GRANULAR CELL NEVUS IN CASE 1

Clinical Findings

One year and a half before initial examination, a 22-year-old man had a right nasal epibulbar, elevated, movable, and exquisitely circumscribed tumor measuring 1.5 mm in diameter near the plica (Figure 1A). The patient believed the lesion had increased in size but not in pigment. There was an eccentric darker brown region that approached the edge but no accompanying flat brown pigmentation in the adjacent conjunctiva. There were several freckles along the superior eyelid margins of both eyes. The patient had a strong family history of cutaneous melanoma. The conjunctival lesion was excised and has not recurred during 7 months of follow-up.

Immunohistopathologic Findings

After fixation in formalin, the excised tissue measured 4 mm × 3 mm × 1 mm. The conjunctival epithelium contained many goblet cells and a small number of junctional nests composed of cohesive, ovoid nevomelanocytic cells that were also in the walls of shallow evaginations of the surface epithelium. Two mildly separated junctional nests that were tightly organized were found beyond the subepithelial portion of the tumor on 1 side. In the subepithelial substantia propria under a thin mantle of collagen was situated a sheetlike collection of unusual polyhedral eosinophilic to granular cells (Figure 1B) occupying the full thickness of the lesion. Common subepithelial neocytes were not found. Focal collections of lymphocytes were found together with a light dispersion throughout the lesion.

The cells were either nonpigmented or faintly pigmented and exhibited a finely to coarsely granular capacious cytoplasm, within which a relatively small nonnucleolated nucleus with finely divided chromatin was
centrally or eccentrically placed, conferring a low nuclear to cytoplasmic ratio; mitotic figures were absent. The deep border was well defined, straight, and noninfiltrative. No mitotic figures were encountered. The reticulin stain revealed the sheathing of individual cells or small clusters by delicate fibers; in contrast, haphazardly distributed fibers were identified within conspicuous collections of lymphocytes. The periodic acid–Schiff stain highlighted the cytoplasmic granularity, which was less dramatically brought out by the Masson trichrome stain; pretreatment with diastase failed to abolish the periodic acid–Schiff positivity. In some tumor cell clusters, the peri-

Figure 1. A granular cell nevus (case 1). A, Exquisitely circumscribed, lightly pigmented, and freely movable epibulbar lesion. B, Large epithelioid, eosinophilic granular cells with small centrally located nuclei are associated with a lymphoid aggregate (left) and diffusely distributed lymphocytes (hematoxylin-eosin, original magnification ×160). C, The principal tumor cells contain large complex and small granules. Goblet cells are present within the epithelium (periodic acid–Schiff after diastase pretreatment, original magnification ×220). D, Subepithelial clusters and individual tumor cells are MART-1-positive. Intraepithelial dendritic melanocytes are also positively stained (immunoperoxidase reaction, diaminobenzidine chromogen, original magnification ×160). E, HMB-45 staining is unequivocal but less intense. No staining is apparent in the epithelium (immunoperoxidase reaction, original magnification ×160). F, Ki-67 staining for intranuclear proliferation protein fails to show a black immunohistochemical product in this typical high-power field. There is a faint cytoplasmic granularity as well as a light brown staining, the latter from the presence of finely dispersed melanin granules (immunoperoxidase reaction, original magnification ×400).
odic acid–Schiff–positive cytoplasmic granules were conglutinated (Figure 1C). Melanophages with coarsely clumped collections of pigment granules were widely scattered beneath the epithelium and among the granular cells.

S100 and MART-1 (Figure 1D) immunostained both the intraepithelial dendritic melanocytes and the small nevocytic clusters within the well-defined junctional cavities. The subepithelial granular cells also stained positively with these 2 probes; the lymphocytes stood out because of their negative staining, but they were CD45-positive. HMB-45 staining was strongly manifested by the granular cells (Figure 1E) but only faintly by the intraepithelial nevocytes within the junctional nests and the intraepithelial dendritic melanocytes. The granular cells were negative for lysozyme, CD45, and CD68 (macrophage/histiocytes) as well as for CD1a (Langerhans cells). Ki-67 immunoreaction revealed conspicuous nuclear staining within the epithelium and scattered lymphocytes, while only exceptionally among the granular cells (Figure 1F). In 2 high-power fields (×400), 259 cells were counted, of which only 3 displayed nuclear immunohistochemical product (proliferation index of 1.2%). Positively staining lymphocytic nuclei in 5 phase exhibited much smaller, rounder nuclei.

**EPITHELIOID CELL (CLONAL OR INVERTED) NEVI IN CASES 2 AND 3**

**Clinical Findings**

The first of 2 patients (case 2) with this lesion was a 19-year-old man who had noted a spot 1 year earlier on the nasal aspect of the surface of his right globe toward the plica. It had grown darker throughout several months. His vision was unaffected. The lesion was uniformly grayish black, 4 mm at the largest diameter, elegantly circumscribed, and freely moveable. The rest of the ocular examination results were unremarkable, and the lesion was removed. There has been no recurrence during 6 months of follow-up.

The second patient (case 3) was a 56-year-old man who became aware of a black spot on the mid-nasal aspect of his right globe approximately 30 years earlier. Throughout several years, the lesion slowly grew, though its color had not changed (Figure 2A). There were multiple small feeding vessels. He had no other ocular complaints. The patient was a carpenter and had experienced considerable sun exposure in his life but had never developed any skin cancers. He was awaiting kidney and liver transplantations as a consequence of long-standing diabetes mellitus and cirrhosis. Visual acuity was 20/20 OU with correction, and intraocular pressure was 12 mm Hg bi- laterally. The conjunctival lesion had a central, elevated, dark to black area surrounded by nonpigmented, more translucent tissue exhibiting a fine intrinsic vascularity. A border of golden brown primary acquired melanosis was not detected. The tumor measured 2.5 mm × 2.0 mm and was freely moveable on the globe. A simple excision with adjunctive cryotherapy was performed. There was no recurrence during 1½ years of follow-up, but the patient died of his systemic diseases.

**Immunohistopathologic Findings**

In the first of the 2 cases, the tissue received in the laboratory after formalin fixation measured 4 mm × 4 mm × 1 mm. A pigmented cellular lesion occupied the vast majority of the conjunctival substantia propria (Figure 2B). Within the goblet cell–rich epithelium were rare, well-defined, lightly pigmented nests of conventional nevocytes. These cells were also in the walls of incipient shallow cysts derived from evaginations of the surface epithelium. Regular nevocytes were encountered in the superficial stroma admixed with lymphocytes, and a prominent collection was observed subepithelially toward 1 edge of the lesion.

The largest part of the lesions in cases 2 and 3 was dominated by variably pigmented, eosinophilic epithelioid cells that were arranged in a dense sheet or else moderately separated from each other either individually or in small clusters by a faintly fibrillar, lightly eosinophilic matrix. The epithelioid cells were endowed with a generally fine dusting of cytoplasmic pigment of low density that never obliterated a clear view of the nucleus (Figure 2C). The latter could be central or eccentric, nucleolated (basophilic but not acidophilic), binucleolate, or multinucleolate, and most characteristically possessed small to extremely impressive intranuclear inclusions of herniated cytoplasm. These imparted a bizarre configuration to the nucleus, which often appeared hyperchromatic owing to compression of the nuclear chromatinic material at the nuclear membrane. A small group of plump spindled cells was also present in the stroma, and numerous melanophages were scattered about. No mitotic figures were discovered.

The formalin-fixed tissue in the second, older patient (case 3) measured 6 mm × 4 mm × 1 mm. It failed to harbor any junctional nests; subepithelial foci of dystrophic calcification within elastotic material, however, were detected. Beneath a prominent subepithelial population of regular nevus cells (type B) were epithelioid cells that were virtually identical to those described in case 2. The stroma between these latter cells was somewhat more sclerotic, and the nuclei were often binucleolated and vacuolated but generally less alarming in shape than those in the first case. The results of the immunostaining for the 2 cases were identical.

S100 was highly positive for the subepithelial epithelioid cells, but less so for the conventional junctional and immediately subepithelial nevocytes in both lesions. MART-1 was dramatically positive for the rare junctional nevus cells, intraepithelial dendritic melanocytes, and subepithelial epithelioid cells (Figure 2D), but more lightly positive for the superficial subepithelial normal-appearing nevocytes. HMB-45 stained most of the epithelioid cells but not the small conventional nevocytes (Figure 2E). CD45 brought out many lymphocytes interspersed among subepithelial nevocytes; far fewer histiocytes were identified with CD68 and lysozyme staining. Ki-67 displayed prominent intraepithelial nuclear staining, particularly along the basilar region that included basal germinal epithelial cells. The small common subepithelial nevocytes and the large epithelioid cells failed to exhibit staining of their nuclei with this marker (Figure 2F).
Figure 2. Epithelioid cell (clonal or inverted) nevi (cases 2 and 3). A, Centrally pigmented lesion with outer rim of translucent tissue (case 3). B, Beneath the goblet cell–rich epithelium are superficial cysts with juxtaposed nevus cells and intermixed chronic inflammation. Eosinophilic epithelioid nevus cells without apparent pigmentation at this power and numerous heavily pigmented melanophages occupy the middle and lower portions of the substantia propria. The deep margin is sharp and noninfiltrating where it abuts the collagenous stroma (hematoxylin-eosin, original magnification x100). C, The epithelioid nevus cells display a moderate dispersion of cytoplasmic melanin granules. Also conspicuous are bizarre single or multiple nuclei with herniations of eosinophilic cytoplasm. No mitotic figures are identified (hematoxylin-eosin, original magnification x400). D, MART-1 highlights 2 intraepithelial nevus cell nests as well as basilar dendritic melanocytes. The cytoplasm of the subepithelial epithelioid cells is strikingly positive beyond the coloration conferred by intrinsic melanization. The scattered lymphocytes are negative (immunoperoxidase reaction, original magnification x100). E, The cytoplasm of many but not all of the subepithelial nevus cells immunostained with HMB-45. Note that the intensity of the immunohistochemical product is greater than that of the discernible pigment in the hematoxylin-eosin–stained section in B, which has been photographed at the same magnification. An intraepithelial junctional nest on the upper left also labels (immunoperoxidase reaction, original magnification x100). F, Ki-67 fails to stain the nuclei with their visible vacuoles. A positive reaction would obliterate the nuclear details with a black product. The brown pigment in the cytoplasm represents melanin (immunoperoxidase reaction, original magnification x400).
**BALLOON CELL NEVUS IN CASE 4**

**Clinical Findings**

A 44-year-old woman who had worked outdoors for many years as a park ranger was examined by her ophthalmologist in the fall of 2008. Ophthalmic examination revealed a moderately brown lesion with foci of darker speckling. It was slightly elevated with an oval shape; movable, smooth, and lustrous; and located on the right episcleral surface near the plica. It measured 6 mm × 4 mm. A smaller contiguous inferior lesion situated in the lower portion of the plica semilunaris was somewhat darker and 1.5 mm at its greatest diameter. Earlier evaluations in 1994 and 2004 had not documented either of the 2 neighboring lesions. There were no discernible cysts, and a fine vascularity was only seen using a slitlamp. There was no flat surrounding pigmentation of primary acquired melanosis. Because of the 2 components of the lesion, it was judged to be suspicious and was removed. There has not been a recurrence during 4 months of follow-up.

**Immunohistopathologic Findings**

The 2 excised fragments of tissue measured 2 mm and 1 mm in greatest diameters. In hematoxylin–eosin–stained sections, the conjunctival epithelium overlying the main component of the lesion did not possess any apparent junctional activity nor dendritic melanocytic hyperplasia; epithelial inclusion cysts were not found. There was a thin collagenous grenz zone containing a moderate number of melanophages that separated the epithelium from variably sized collections of small standard nevus cells occupying the superficial subepithelial substantia propria (Figure 3A). The latter cells were superficially arranged in a sheet, while the deepest layers were composed of spindle cells that were lightly stromatized and incompletely excised. Melanophages were minimally dispersed throughout the lesion, which was for the most part nonpigmented.

The most arresting feature of the lesion was a population of polygonal, granular, clear, or ballooned cells located in the middle and central subepithelial zones (Figure 3B). A generally centrally located, small nucleus contained an inconspicuous nucleolus; the nuclear chromatin was delicate and finely divided. Many of the nuclei had intranuclear sequestrations of cytoplasm and indentations of the nuclear membrane; some cells displayed larger nuclei or binucleation. Periodic acid–Schiff staining of the clear cells failed to reveal any cytoplasmic granularity. No mitotic figures were discovered. At the edges of the specimen near these balloon cells were conventional nevocytes that were densely packed with slightly angulated, small nuclei that occasionally exhibited intranuclear vacuoles but no nucleoli. Also interspersed among this population and at its outskirts were clear balloon cells that resembled those in the center of the lesion. The satellite, or plical portion, of the mass was composed of small subepithelial nevus cell nests or single cells exhibiting a perinuclear clear area and an outer submembranous rim of cytoplasmic melanin in the shape of a ring, suggesting transitional cells. The overlying epithelium had 2 well-defined junctional nests.

Immunostaining with S100 and MART-1 (Figure 3C) highlighted all populations of nevus cells, including the totally clear cells (balloon cells); also positively reacting were abundant intraepithelial dendritic melanocytes and the nevocytes in the rare junctional nests, particularly those located at the edge of the tumor. HMB-45 did not stain the subepithelial balloon cells nor the conventional intraepithelial and subepithelial nevocytes; the intraepithelial dendritic cells stained faintly positive. MART-1 showed that the intraepithelial dendritic cells, while individually disposed along the basal epithelial region, were obviously hyperplastic; this feature was not seen in hematoxylin-eosin–stained sections. Ki-67 highlighted the nuclei of many positively stained basal epithelial cells but none in the cells constituting the junctional nests (Figure 3D). Neither the balloon cells nor the conventional subepithelial nevocytes stained positively. The balloon cells were negative for lysozyme, CD45 antigen, and CD68 antigen used for the identification of histiocytes/macrophages.

**BLUE NEVUS IN CASE 5**

**Clinical Findings**

A 54-year-old man became aware of a left conjunctival pigmented lesion 2½ years earlier on the superonasal surface of his right globe. It had recently darkened over several months and also doubled in size with the acquisition of a minimal but perceptible thickness (Figure 4A). It measured 3 mm × 4 mm; was flat and uniformly pigmented; was situated several millimeters away from the limbus; had irregular feathery edges; and lacked cysts, a feeder vessel, and any associated surrounding flat, golden brown pigmentation. Fine blood vessels in the conjunctival substantia propria and episclera were outlined by the pigmentation. The conjunctiva moved freely over the pigmented spot, while the latter was fixed to the sclera and immovable. Vision was 20/20 OU, and the tension by applanation tonometry was within normal levels in both eyes. Ultrasound biomicroscopy and gonioscopy failed to demonstrate an underlying mass in the ciliary body or chamber angle. A small, finely vascularized, nonpigmented stromal thickening at the root of the blue iris was diagnosed as an epithelium or small nevus. Several small, flat pigmented nevi of the right fundus were discovered on dilated fundus examination. These uveal findings suggested a forme fruste of ocular melanocytosis. The epithelial lesion was excised down to the scleral plane and has not recurred during 1½ years of follow-up.

**Immunohistopathologic Findings**

After formalin fixation, 2 fragments of tissue measured 5 mm × 5 mm × 1 mm and 3 mm × 2 mm × 1 mm. There was no detectable intraepithelial melanocytic hyperplasia. The superficial sclera and the deep substantia propria contained loosely arranged spindled and occasionally dendriticiform cells set in a variably collagenized but immovable. Vision was 20/20 OU, and the tension by applanation tonometry was within normal levels in both eyes. Ultrasound biomicroscopy and gonioscopy failed to demonstrate an underlying mass in the ciliary body or chamber angle. A small, finely vascularized, nonpigmented stromal thickening at the root of the blue iris was diagnosed as an epithelium or small nevus. Several small, flat pigmented nevi of the right fundus were discovered on dilated fundus examination. These uveal findings suggested a forme fruste of ocular melanocytosis. The epithelial lesion was excised down to the scleral plane and has not recurred during 1½ years of follow-up.

After formalin fixation, 2 fragments of tissue measured 5 mm × 5 mm × 1 mm and 3 mm × 2 mm × 1 mm. There was no detectable intraepithelial melanocytic hyperplasia. The superficial sclera and the deep substantia propria contained loosely arranged spindled and occasionally dendriticiform cells set in a variably collagenized but generally loose stroma without a well-defined, tight fascicular pattern and with indistinct margins (Figure 4B). The cells endowed with less pigment displayed a deli-
cate diaphanous cytoplasm and were faintly pigmented (Figure 4C). Those that were more superficially located contained a greater complement of cytoplasmic melanin granules that did not obscure identification of their banal nuclei. The latter tended to be small and widely separate from one another; punctate nucleoli and small intranuclear vacuoles (herniations of cytoplasm) were readily apparent. Lymphocytes were absent; melanophages with large, coarsely clumped collections of melanin granules concealing the nucleus were present among the superficial, more heavily pigmented nevus cells. No mitotic figures were observed. Immunostaining for Melan-A (analogous to MART-1) (Figure 4D) and PNL2 (analogous to HMB-45), with only the red chromogen aminoethylcarbazole used in this case, was positive. Ki-67 immunostaining demonstrated nuclear positivity in only 1 of 176 cells counted in 2 high-power fields (×400) (proliferation index of 0.6%). A summary of the preceding immunohistochemical staining results of the 4 categories of lesions comprising this series is presented in Table 2.

COMMENT

None of the lesions in this series was associated with primary acquired melanosis, the most common precursor of conjunctival melanoma. Four of 5 lesions were morphologic variants of the common acquired conjunctival nevus that begins in the epithelium as a nevomelanocytic proliferation of junctional nests (theques) and progressively evolves as the patient ages into compound or completely subepithelial stages (as exemplified by our oldest patient, aged 56 years, case 3), accompanied by
variably prominent epithelial inclusion cysts. Junctional nests did not extend radially far beyond the subepithelial nevoid component. Subclinical microscopic epithelial inclusion cysts were detected in 3 lesions. The blue nevus, on the other hand, represents an arrest in the migration of neural crest–derived melanocytes that come to reside in the connective tissue and never reach the epithelium. In this series, the blue nevus was the only immovable lesion owing to its location in the superficial sclera rather than the conjunctival substantia propria.

We describe for the first time the results obtained from the simultaneous application of a panel of 3 probes (dis-

**Figure 4.** A blue nevus (case 5). A, Juxtalimbal brown, immovable lesion with feathery edges. B, Superficial scleral lamellae are infiltrated by a banal and hypocellular population of lightly pigmented spindle cells. There is heavier pigmentation superficially (hematoxylin-eosin, original magnification × 100). C, The nuclei are delicate and frequently exhibit vacuoles. The stroma is loose and delicately fibrillar (hematoxylin-eosin, original magnification × 160). D, Melan-A–positive staining (same as MART-1) of the spindled melanocytes (immunoperoxidase reaction, aminoethylcarbazole chromogen; original magnification × 300).

**Table 2. Results of Immunolabeling**

<table>
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<tr>
<th>Nevus Type</th>
<th>S-100</th>
<th>MART-1</th>
<th>HMB-45</th>
<th>CD1a</th>
<th>CD68</th>
<th>Ki-67, % of Immunostained Nuclei of Total Cells</th>
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<td>Granular cell nevus</td>
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<td>+++</td>
<td>+</td>
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<td>0</td>
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<tr>
<td>Balloon cell nevus</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>Blue nevus</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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</tr>
</tbody>
</table>

Abbreviations: ND, not done; –, negative; +, light; ++, moderate; ++++, heavy.

a Proliferation index.
b A subpopulation of common subepithelial nevocytes were also present and stained S-100 (+++), MART-1 (+++), HMB-45 (−), and Ki-67 (−).
c PNL2 staining was performed instead and was moderately positive.
tint from a single probe) to a subset of conjunctival nevi. These markers are of proven value for the identification of cells of melanocytic origin, namely, antibodies against S100 protein, MART-1, and HMB-45. Additionally, immunolabeling for the Ki-67 nuclear proliferation protein was undertaken. S100 stains both the nucleus and cytoplasm. This 21-kDa moiety was first discovered in glial cells and was termed S-protein owing to its solubility in a supersaturated solution of ammonia sulfate. A role has been imputed to it in guiding cytoplasmic calcium fluxes or microtubular assembly. The alpha/alpha dimer of S100 protein is preferentially expressed in melanocytes. S100 protein is less specific than premelanosomal antigens, and many nonmelanocytic cell types can express it. It retains a special role in diagnosing desmoplastic melanomas, in which MART-1 and MMB-45 antibodies are often nonreactive. MART-1, its close relative or twin Melan-A, and HMB-45 are intracytoplasmic epitopes of melanosomal-related antigens that have been extensively used in studies of cutaneous nevi and melanomas, but with less fruitful current results in conjunctival investigations. Antibodies against these antigens actually react with a premelanosomal group of glycoprotein antigens referred to as gp100 (100 for kilodaltons). HMB-45 is at the gp10 (kilodalton) end of the complex and has been regarded as a cytoplasmic marker for melanocytic activation that supposedly suggests a more suspect cytologic composition when diffusely present within most of the cells of a given lesion. Ki-67 immunostaining detects a nuclear proliferation protein that is preferentially expressed during late G1, S, M, and G2 phases of the cell cycle, while cells in the G0 (quiescent) phase are negative. It is far more sensitive for estimating proliferation than counting mitotic figures. This marker has an accepted role when coupled with histopathologic evaluation in distinguishing benign from malignant cutaneous melanocytic lesions. To date, it has been sporadically but not systematically used in the evaluation of conjunctival lesions whether they are routine or atypical, combined, spindle cell, dysplastic, inflamed, juvenile, Spitz, or borderline nevi.

Case 1 is a granular cell nevus, the first ever recognized in the conjunctiva. A single report of 2 cutaneous examples has recently appeared in the dermatopathology literature. The initial pathologic impression was that the lesion represented an unusual histiocytic reaction, which was reinforced by the presence of a prominent lymphocytic infiltrate. The constituent cells were polygonal or polyhedral and had a distinctive eosinophilic granular cytoplasm that differed from the classically glassy cytoplasm of epithelioid histiocytes and epithelioid melanoma cells. Their nuclei were generally small and centrally located and displayed punctate nucleoli. The granules were sometimes conglutinated and were lightly positive on Masson trichrome staining but exhibited vivid periodic acid–Schiff–positive, diastase-resistant staining. Negative CD45, CD68, CD1a, and lysozyme staining ruled out the possibility that the epithelioid cells were of monocytic/histiocytic or Langerhans derivations. S100, MART-1, and HMB-45 immunolabeling in the current case was uniformly strongly positive throughout the entire lesion, keeping with the results described in the 2 cutaneous cases; HMB-45 is either negative or lightly positive only in the junctional cells of common compound nevomelanocytic nevi. Ki-67 positivity was detected in only 3 of 259 cells counted in 2 high-power fields (×400) of our conjunctival lesion (unfortunately, it was not determined in the evaluation of the 2 cutaneous examples). This is a proliferation index of around 1% and highly suggestive of a benign diagnosis.

The 2 epithelioid cell nevi in this series are the most likely to be misdiagnosed as melanoma and are the first examples to be fully characterized in the conjunctiva. The first of our 2 patients' lesions displayed rare junctional nests but was predominantly subepithelial, while the other tumor in an older patient was completely subepithelial. These tumors are also referred to as clonal nevi because of the striking population of subepithelial epithelioid cells that coexist with conventional small nevus cells. Clonality in this context simply refers to a shared morphology among the cells and not to any genetic identity. Inverted type A nevus is another term that has the virtue of highlighting the location of the large epithelioid cells in the middle or lower dermis of the skin or the same levels of the conjunctival substantia propria, where small type B nevus cells are expected to be present. An earlier conjunctival study published prior to the report of this condition in the skin probably described a related case. A similar inverted phenomenon can occasionally be observed in juvenile conjunctival nevi.

These epithelioid tumors displayed the most alarming cytologic features, consisting of an abundance of cytoplasm containing a fine dispersion of cytoplasmic pigment granules and possessing bizarre nuclei that often displayed binucleation or multinucleation. These cells were juxtaposed to regular nevocytes and arranged in either sheets or aggregated into small clusters separated by a lightly fibrillar or early sclerotic eosinophilic stroma. Their nuclei displayed multiform shapes and were often dominated by large vacuoles (actually herniations of cytoplasm); mitotic figures were not discovered. The deep margin was circumscribed and noninfiltrative. S100, MART-1, and HMB-45 immunostaining was positive in the cytoplasm of the epithelioid cells, which also showed immunonegativity for CD45, CD68, and lysozyme; the small collections of common subepithelial nevocytes in both lesions were S-100– and MART-1–positive but HMB-45–negative. One lesion manifested a moderate subepithelial lymphocytic infiltrate. Ki-67 nuclear immunolabeling was totally negative, which reinforced the interpretation of a benign lesion.

Benign and malignant balloon cell melanocytic neoplasms are well recognized in the skin, are usually lightly pigmented to nonpigmented, and generally occur in younger individuals. They constitute 2.0% of cutaneous nevi but are exceedingly rare in the conjunctiva. Shields et al reported none among 410 consecutively excised nevi at the Wills Eye Institute. At least 6 previous cases of conjunctival balloon cell nevi have been described. Our balloon cell nevus was composed of equal parts conventional nevocytes and polygonal, clear, vacuolated, and mostly mononucleated but rarely binucleated cells that were periodic acid–Schiff–negative. The nuclei were orthocho-
matic, small, centrally located and exhibited minute nucleoli. The cells measured approximately 20 to 25 µm in diameter; because of their large size and clear cytoplasm, they could be mistaken for xanthoma cells, adipocytes, or a metastasis from a renal cell carcinoma.

Cutaneous and conjunctival balloon cells are created by collections of malformed vesicular premelanosomes51,52 and not, as has been suggested by some, by the accumulation of cytoplasmic lipid. The conjunctival balloon cells in our case were S100- and MART-1-positive but HMB-45-negative; CD45, CD68, and lysozyme negativity ruled out a histiocytic lineage; and Ki-67 immunostaining was entirely negative, indicating no proliferative tendency. In 2 earlier articles59,60 with immunohistochemical evaluations, the conjunctival balloon cells were S100-positive, though negative for HMB-45, lysozyme, and CD68, as observed in our case. Based on their MART-1 positivity and HMB-45 negativity, the balloon nevus cells parallel the staining results of conventional subepithelial nevus cells more closely than those comprising the granular and epithelioid cell nevi described above, both of which were MART-1- and HMB-45-positive.

When encountered in the conjunctiva, a “blue” nevus is actually brown, unlike in the skin, where the Tyndall effect preferentially reflects the blue wavelengths. While rarer in the conjunctiva than in the skin,23 blue nevi are probably the second most common nevus type in the conjunctiva54,55 after common acquired nevomelanocytic nevus, accounting for 1% of 410 excised nevi in the previously mentioned series.60 The blue-brown nevus in this study is the first epibulbar example to be evaluated with immunohistochemical markers. It was located in the superficial sclera and deep substantia propria. The lesion was microscopically hypocellular (thereby differing from extrasceral extensions of uveal melanomas) due to a delicate enveloping stroma. It was constituted by fusiform or dendritic cells that were lightly pigmented or nonpigmented at its center and more pigmented at the superficial periphery; the pigment was never so coarsely clumped that it obscured the nucleus except in a few melanophages. The nuclei were delicate and occasionally displayed intranuclear cytoplasmic sequestrations and punctate nucleoli; no mitotic figures were found. Immunostaining was positive for MART-1 and PNL2 (the latter is a newer antimelanocyte monoclonal antibody that shares many of the immunolabeling properties of both MART-1 and HMB-45, including positivity for common nevocytes and blue nevus cells57). The Ki-67 proliferation index was less than 1%. In contrast, episcleral extensions of uveal melanomas are typically percellular and have a proliferation index of around 20%.58 When evaluating the relative merits of the various immunohistochemical probes used in this investigation, one should remember that S100 immunoreactivity, while highly sensitive, unfortunately indiscriminately stains both benign and malignant melanocytic lesions as well as a handful of other cell types.7,8,25,26 Previously reported immunohistochemical studies of cutaneous melanocytic lesions analyzed with MART-1 and HMB-45 have established that these probes also cannot decisively separate benign from malignant tumors.8,10,16,20,22 HMB-45 has been advanced as a marker of cellular activity or proliferation in the skin7,28 and conjunctiva16,18,20 and is expressed in melanomas more often and more intensely than in nevi.5,16,18,20,25-27 Common conjunctival nevus cells have been reported to be positive for HMB-45 in 8.5% to more than 50% of lesions.16,17 Steuhl and associates28 and Sharara and colleagues29 have accurately underscored the restriction of this staining to the junctional region. MART-1 has not been previously evaluated and contrasted with HMB-45 in conjunctival lesions. Only 2 brief and incomplete studies23,24 have described low counts of Ki-67 immunoreactivity in conjunctival nevi, but did not clearly state the immunostaining results with this marker in melanomas. In the skin, melanomas are frequently MART-1- and HMB-45-positive, with a high proliferation index (10%-70%) using Ki-67 antibodies7,15; nevi typically exhibit MART-1 positivity, HMB-45 negativity, and a proliferation index of 2% or less.9,10,12,24,45

The 4 atypical types of conjunctival nevi delineated in this study yielded immunohistochemical findings supportive of a benign diagnosis. Beyond the observations that the granular and epithelioid cell nevi both stained strongly positive for MART-1 and were aberrantly positive for HMB-45 throughout all levels of the lesions, they more importantly displayed negligible to no Ki-67 positivity among the cells in the substantia propria. We have inferred from our data that HMB-45 positivity among variant nevus cells represents an abnormality in melanogenesis rather than a marker of cellular activation and therefore does not necessarily signal a menacing biologic potential. Currently, Ki-67 nuclear immunostaining for determining the proliferation index supersedes the diagnostic value of the differential expression of melanocyte-specific antigens. A Ki-67 proliferation index of less than 2% is most compatible with a nevus, whereas a result greater than 10% strengthens the diagnosis of a melanoma9,12,13,43

A final caveat should be offered: When counting Ki-67 immunolabeled nuclei in melanocytic lesions, care must be taken not to enumerate nuclei belonging to lymphocytes, histiocytes, vascular endothelial cells, or epithelial germinal cells participating in inclusion cysts. CD45 positivity of aggregates or a light dispersion of S-100 or MART-1-negative cells for positive ascertainment of lymphohistiocytic cells; CD31 and CD34 for vascular endothelial cells; and cytokeratin cocktail for squamous epithelium should defend against these pitfalls.

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